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COLONY-FORMING UNIT REPOPULATION AND SPLIT-DOSE RADIOSENSITIVITY IN ENDOTOXIN TREATED AND CONTROL LAF, MICE

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ABSTRACT

Radiosensitivity of an animal, in terms of survival or death following midlethal exposure, is thought to be related to the surviving number of hematopoietic stem cells. After a sublethal exposure to radiation an animals' sensitivity to a subsequent exposure (LD_{50}) might also be expected to be related to the number of stem cells which are present at any given time. In the present experiments with mice, we have studied the relationship between split-dose LD_{50} and changes in the numbers of nucleated cells in the femur and the femoral content of colony-forming units (CFU's). These CFU's are proliferative cells in the marrow which when transplanted have the capacity to form nodules in the spleens of supralethally irradiated recipient mice. Many stem cell-like attributes have been conferred to CFU's, and the CFU is frequently referred to as a hematopoietic stem cell.

In the present experiments, changes in marrow cellularity and CFU content were studied for three weeks after exposure to 450 R, and preliminary LD_{50}'s were determined at 5 or 14 days. One group of animals was given bacterial endotoxin before the 450 R exposure. During the first week after 450 R, the endotoxin-treated animals showed an accelerated recovery in terms of numbers of nucleated marrow cells and CFU's, and at day 5 the femoral CFU content of the endotoxin-treated animals was ten times as great as that of the controls. However, at this time the LD_{50} of the endotoxin-treated animals was only 50 R higher than the

controls. At 14 days, the femoral CFU content of the control animals was approximately four-fold greater than that of the endotoxin-treated group, but the LD_{50}'s of the two groups were quite similar. Therefore, in these experiments with endotoxin-treated and control animals, the femoral CFU content and split-dose radiosensitivity have been found to vary independently.

SUMMARY

The Problem:

After an initial sublethal exposure to radiation, the sensitivity of animals to a second exposure (LD_{50}) is thought to be related to the degree of injury remaining to the bone marrow, or conversely, the extent to which "recovery" of the bone marrow has occurred after the initial exposure. If a means were available by which the degree of injury to the marrow or the extent of recovery of the marrow could be measured at any given time, the mortality expected from a second exposure could be predicted.

The Findings:

The numbers of nucleated bone marrow cells and the numbers of colony-forming units (CFU's) per femur have been measured at various times after 450 R in endotoxin-treated and control mice. The LD_{50}'s have also been measured at 5 and 14 days after 450 R. The data show that although the femoral CFU content of endotoxin-treated mice was ten times that of controls at 5 days, the LD_{50} of the endotoxin-treated group was only 50 R higher. At 14 days, the femoral CFU content of the control animals was four times that of the endotoxin animals but the LD_{50}'s were similar. These data indicate that CFU content of the femur and LD_{50} vary independently, and that the content of CFU's, which have been called the hematopoietic stem cells, does not predict sensitivity to a second radiation exposure.

INTRODUCTION

When mice are given midlethal exposures to X or γ radiation, the resulting syndrome is characterized by injury to the bone marrow and a marked decrease in the numbers of leukocytes and other cellular elements in the peripheral blood (1). The pancytopenia is ultimately attributed to radiation injury to the hematopoietic stem cell, that is, the killing of stem cells (1). Whether an animal survives or succumbs following midlethal exposure is influenced by the surviving number of stem cells and the rate at which stem cells and their progeny proliferate and repopulate the hematopoietic system (1).

Recuperation of a cell population from radiation injury appears to be divided into at least two phases: (1) the first 24-48 hours during which cellular or molecular repair of sublethal injury occurs, and (2) subsequent proliferation of the surviving cells These recuperative processes occur in irradiated cells cultured *in vitro* (2, 3, 4) and they may also occur in hematopoietic stem cells irradiated *in vivo* (5, 6).

Methods are now available that enable quantitation of a group of marrow or spleen cells which, after transplantation, have the capacity to proliferate and form nodules or colonies in the spleens of supralethally irradiated recipient mice (7, 8). These colony-forming units (CFU's) are thought to be hematopoietic stem cells (1) although this has not been conclusively demonstrated. Assuming that the size of the hematopoietic stem cell population plays a dominant role in determining

radiosensitivity of an animal following sublethal irradiation, the extent of stem cell proliferation might be expected to correlate with, or even predict, the sensitivity to a second radiation exposure. If the CFU is the stem cell or if the number of CFU's is directly related to the number of stem cells, the number of CFU's at any given time should predict, at least qualitatively, the sensitivity of an animal to a second radiation exposure following sublethal irradiation.

An earlier report dealt with the extent to which the numbers of CFU's in the marrow or the numbers of endogenous CFU's in the spleen of mice could be correlated with the radiation-protection produced by bacterial endotoxin (9). Those data showed that in certain respects the numbers of CFU's did qualitatively correlate with the extent of radiation protection. However, by varying the time of endotoxin injection relative to irradiation, a procedure which influences both the extent of protection and the number of endogenous CFU's, it was shown that radiation-protection and spleen CFU content varied independently. Recently, Smith, et al. reported similar findings (10).

In the present experiments we have used the method of bone marrow transplantation to determine the extent to which the number of femoral CFU's correlate with split-dose radiosensitivity in endotoxin-treated and control mice.

MATERIALS AND METHODS

The animals used were female LAF_1 mice between 3 and 4 months of age which were bred in this laboratory. They were placed in lucite tubes

which were placed on a rotary turntable for whole-body irradiation with 250 kvp X rays. The TSD was 40 inches, HVL 1.49 mm of Cu and the dose rate was 28 to 30 R/min. Endotoxin used in these experiments was PIROMEN, a highly purified Pseudomonas polysaccharide (PP) which was supplied by Flint Eaton and Company. In all experiments a dose of 50 micrograms was given intravenously in a volume of 0.05 cc.

Bone marrow was obtained by methods previously described (9). The marrow preparations were counted and appropriate dilutions injected into supralethally (900 R) irradiated recipients to determine CFU/10^5 cells. The irradiated recipients were housed singly, and their spleens were harvested 8 days after injection. Spleen nodules greater than .24 mm were counted with a dissecting microscope and from these data total nucleated cells, total femur CFU content and CFU's/10^5 nucleated cells were determined. The CFU counts have not been corrected for the fraction of injected CFU's which do not produce nodules in the spleen.

Two groups of mice were used for single dose $LD_{50/30}$ determinations. One group of mice received 50 µg of endotoxin intravenously 24 hours before irradiation, the other served as non-injected controls. Survival was recorded for 30 days. Median lethal doses ($LD_{50/30}$'s) and other statistics were computed by methods previously described (9).

Recovery from the initial radiation injury was studied by the classical split-dose technique. Groups of control and endotoxin-treated mice received an initial or conditioning exposure of 450 R (~ 2/3 of the control $LD_{50/30}$). These animals were then divided into several groups

and were re-exposed at 5 or 14 days after the conditioning exposure to determine the LD_{50}. The difference between the single exposure LD_{50} and the redetermined LD_{50} represents an estimate of the amount of injury remaining from the conditioning exposure at the time tested.

RESULTS

The bone marrow cellularity, the total colony-forming unit (CFU's) content per femur, and the CFU content per 10^5 nucleated cells were measured before and at various times after 450 R. These measurements were made in both endotoxin-treated (24 hours before 450 R) and control mice at the various times. Figure 1 shows the changes in nucleated cell content of the femur and total CFU content of the femur of endotoxin and control mice. The nadir in nucleated cell count occurred at 3 days with the endotoxin-treated mice showing a slightly less depression than did the controls. Between 4 and 7 days the nucleated cell counts in the treated animals were somewhat higher than in the controls, and at 11 days the cellularity had returned to normal with some tendency towards an overshoot in the control animals.

Figure 1 also shows that the femurs of endotoxin-treated mice contained about 70% more CFU's than did the controls at the time of irradiation. Following the initial fall the treated animals appeared to initiate proliferation between 24 and 48 hours after irradiation while in control mice the initiation of proliferation began between 48 and 72 hours. The CFU content remains higher between days 1 and 7 in the treated animals and a peak is reached 5 days after irradiation in the endotoxin

group. At that time they contained ~ 10 times as many CFU's as did control femurs (~ 1900 vs. ~ 200). Subsequently there was an apparent reduction in the total CFU content in the endotoxin-treated mice, whereas, there was continuing proliferation in controls. The control mice subsequently showed a 60% overshoot at 14 days which was not seen in the endotoxin group, and both were near normal 23 days after irradiation.

Figure 2 shows the changes in CFU content per 10^5 nucleated femur cells. This figure shows the relationship between changes in nucleated cell count and in CFU content. During any period when the CFU content increases proportionally to the nucleated cell content the curve would be flat, such as between 3 and 9 to 11 days in the controls and between 9 and 14 days in the endotoxin-treated mice. In both groups, the numbers of CFU's/10^5 cells fell to approximately the same level at 24 hours after irradiation, and the treated animals showed an increase in CFU's that precedes the controls by about 24 hours. A peak is seen in endotoxin-treated animals 5 days after irradiation. This was succeeded by a secondary fall and a return to normal at 23 days. In controls, there was a rapid increase in CFU concentration during days 2 and 3, followed by a plateau between days 3 and 7 at which time the CFU content was about 30% of normal. This was followed by a slower rise to the normal range 14 days after irradiation.

Table 1 contains the single exposure LD_{50} determinations and the preliminary split-dose LD_{50} determinations for endotoxin and control mice. Twenty-four hours after endotoxin, the single exposure LD_{50} was raised by

217 R (924 R vs. 707 R). Five days after 450 R the LD_{50} for the endotoxin-treated group was significantly higher than controls (672 R vs. 617 R). Fourteen days after 450 R, the LD_{50}'s for the two groups did not differ significantly.

DISCUSSION

In the present experiments we have measured the changes in nucleated cell content and in the colony-forming unit (CFU's) content in the femurs of sublethally irradiated mice. A comparison has been made between endotoxin-treated and non-treated mice, the object being to evaluate the relationship between recovery of the bone marrow CFU content and split-dose radiosensitivity.

The data show that endotoxin (PP) given 24 hours before 450 R increased the marrow CFU content at the time of irradiation. This finding is in contrast to the findings of Smith, et al. in which they report no increase in CFU content after treatment with an endotoxin derived from S. typhosa (11). The 70% increase in CFU's was not accompanied by a detectable increase in the nucleated cell content (marrow cellularity). The post-irradiation fall in CFU's was more rapid than the fall in total marrow cellularity. The fall in nucleated cell count is probably the combined result of reduced production, cell death, and loss of cells through maturation and release into the circulation. The extent to which release from the marrow influences the fall in CFU content in either the treated or the control group is not known, although CFU's are normally released from the bone marrow at a constant rate (12, 13) and endotoxins may increase the rate of release (9).

The total fall in CFU content per femur at one day is of some interest. The fall in the PP group was less than in the control group and this may be the result of the slightly higher femur content of CFU's in the PP animals at the time of exposure. In both the PP and control group the surviving fraction, relative to the CFU count at the time of irradiation fell to ~ .003. Assuming an extrapolation number of 2, this surviving fraction would indicate a D_0 of 88 R. This is well within the range of radiosensitivity ascribed to the CFU (7, 8, 9).

The increase in femoral CFU content began about 24 hours earlier in the PP animals than in the control animals, and in both groups the increase in CFU content preceded the increase in marrow cellularity by a day or two. During the first 5 days the rate of increase in CFU content may be higher in the PP than in the control animals, and this is also the case for marrow cellularity between days 3 and 7. Such an early increase in marrow cellularity and CFU's in endotoxin-treated animals has been reported recently by Smith, et al. (11). The endotoxin-treated mice may well begin their proliferation sooner as a result of the stimulation of the CFU compartment by the endotoxin injection prior to irradiation.

Figure 2 shows that in endotoxin-treated mice the increase in CFU's was relatively greater than the increase in marrow cellularity during the first 7 days after 450 R. In contrast, the control animals showed an increase in CFU's which was more or less proportional to the increase in marrow cellularity between 3 and 11 days after irradiation. This

figure also shows a decrease after day 7 in the number of CFU's/10^5 marrow cells. The reason for this decrease is not known, but any of the following factors could produce this effect: depletion of the population from which CFU's are produced, response to feed-back inhibition, migration from the marrow, maturation into non-proliferative cells, or the decline may be indicative of "abortive regeneration" in the CFU compartment.

At 11 and 14 days an "overshoot" in marrow cellularity was observed in both groups, and an overshoot in CFU's was observed in the controls but not in the PP animals. During this time the CFU content in the PP animals was lower than in the controls, the PP animals thus behaving as if the earlier production of CFU's was at the expense of CFU content at 11 and 14 days.

The present data offer a unique opportunity to determine the extent to which CFU content or nucleated cell content can be correlated with split-dose radiosensitivity. If the CFU is the hematopoietic stem cell or if at any given time the CFU content is directly related to the size of the stem cell population, then animals with a higher CFU content would be expected to have a higher LD_{50}. The same would be the case for nucleated cell content if it is assumed that an increase in the nucleated cell content is indicative of an increase in the stem cell content. Such an assumption of a direct relation may be readily challenged on theoretical grounds, depending upon the relationship which is assumed between repopulation of the stem cell compartment(s) and the numbers of cells which

move into the dividing and maturing compartment(s).

The present split-dose LD_{50} data, although preliminary, indicate that split-dose radiosensitivity and femoral CFU content or marrow cellularity vary independently. At 5 days after 450 R the femoral CFU content in the PP animals was about 10 greater than in the controls and the marrow cellularity was about 3-fold greater. At this time the LD_{50} of the PP animals was 55 R higher than in the controls which is markedly lower than the ~ 200 R difference predicted by a ten-fold difference in the number of CFU's with a D_{37} of ~ 90 R. At 14 days the LD_{50}'s for the PP and control animals were essentially the same, although the CFU content was about 3-fold greater in the control animals than in the PP animals, and the marrow cellularity was ~ 50% greater in the PP animals. One further inference may be drawn regarding the relationship between recuperation from radiation injury, as evaluated by the split-dose technique and femoral CFU content. In mice the rate of recuperation is such that 50% of the injury is repaired by 2-4 days after an acute exposure (6). In contrast, the present data show that the femoral CFU content does not approximate 50% of normal in control mice until ~ 9 days. At 5 days the animals had recuperated from ~ 80% of the initial injury, and at that time the femoral CFU content was ~ 5% of normal. At 14 days the animals showed recuperation from ~ 90% of the initial injury, whereas, the CFU content was 50% above normal. Therefore, the rate of recuperation is more rapid than is the rate of CFU repopulation of the femur.

The value and widespread application of the various spleen CFU techniques for studying the various radiobiological and physiological

responses are well established (1, 7, 8, 12, 14, 15). A logical outgrowth of these findings has been to equate the CFU and the bone marrow stem cell, or to assume that changes in the numbers of CFU closely reflect changes in stem cell populations. The present data indicate that with the methods available now, and the inherent problems in the methodology and interpretation, measurable changes in CFU content of the bone marrow do not correlate with variations in split-dose radiosensitivity, and therefore may not closely reflect changes in the stem cell population. The following are some of the factors which may have bearing: (a) The CFU may not be related closely enough to the "stem cell" to accurately reflect the changes in this cell population. (b) The bone marrow "stem cell" and CFU may be closely related or in fact the same, but are not the principle factor in determining split-dose radiation sensitivity. (c) After an initial exposure the bone marrow stem cells or CFU's may have different physical characteristics which prevent an accurate assessment of their total numbers by the commonly used transplantation techniques.

Studies are continuing which may permit us to better understand repair and recovery processes in the bone marrow and the influence(s) of endotoxin on these processes.

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TABLE 1

THE EFFECT OF ENDOTOXIN ON SINGLE AND SPLIT-DOSE LD_{50}

Treatment	Number of Mice	$LD_{50/30}$
Single Exposure		
Endotoxin[a]	79	924 (915-936)[b]
Controls	130	707 (691-723)
Split Dose[c]		
5 Day Endotoxin	55	672 (569-687)
Control	58	617 (597-642)
14 Day Endotoxin	50	680 (665-700)
Control	70	673 (658-693)

[a] 50 μg 24 hours before irradiation.

[b] Brackets include 95% confidence limits.

[c] Initial exposure 450 R; 50 μg of endotoxin given before the initial exposure only.

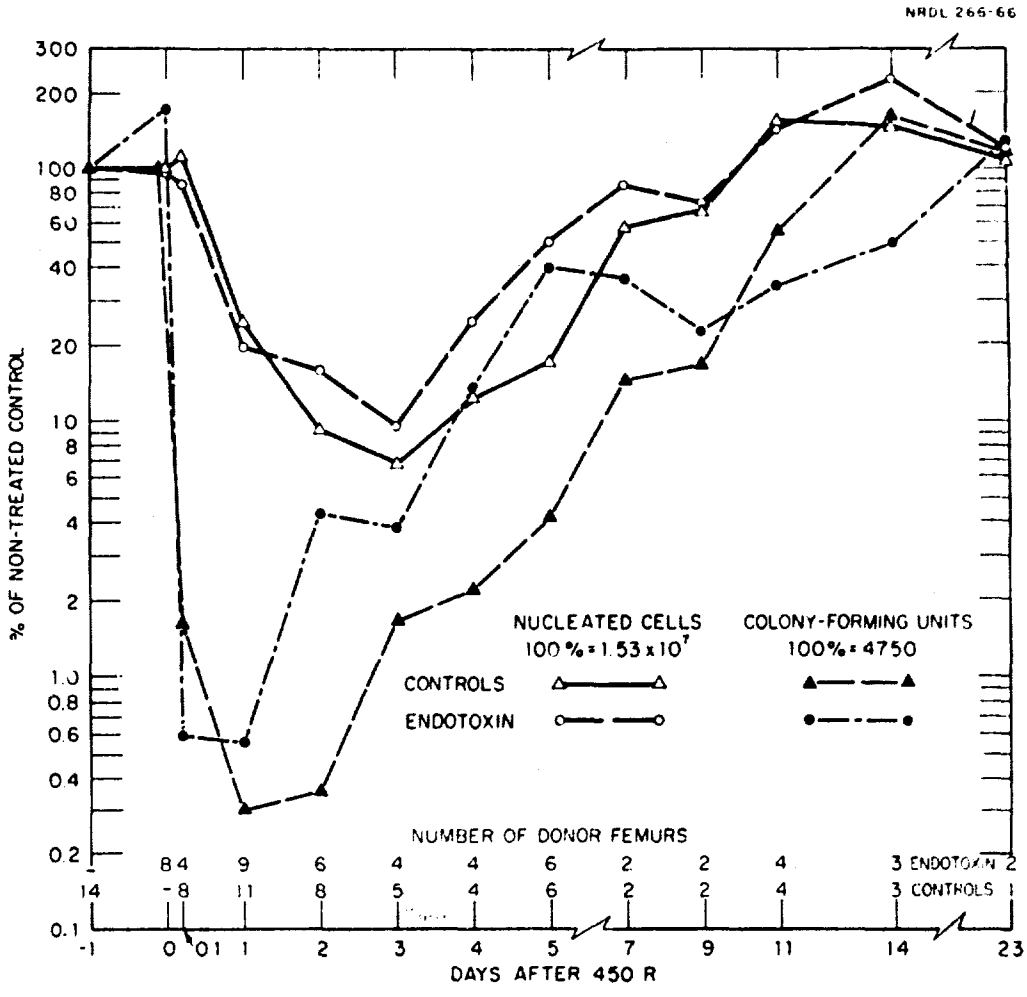

Fig. 1. Time-dependent changes in the numbers of nucleated cells and colony-forming units in the femur of endotoxin-treated or control mice.

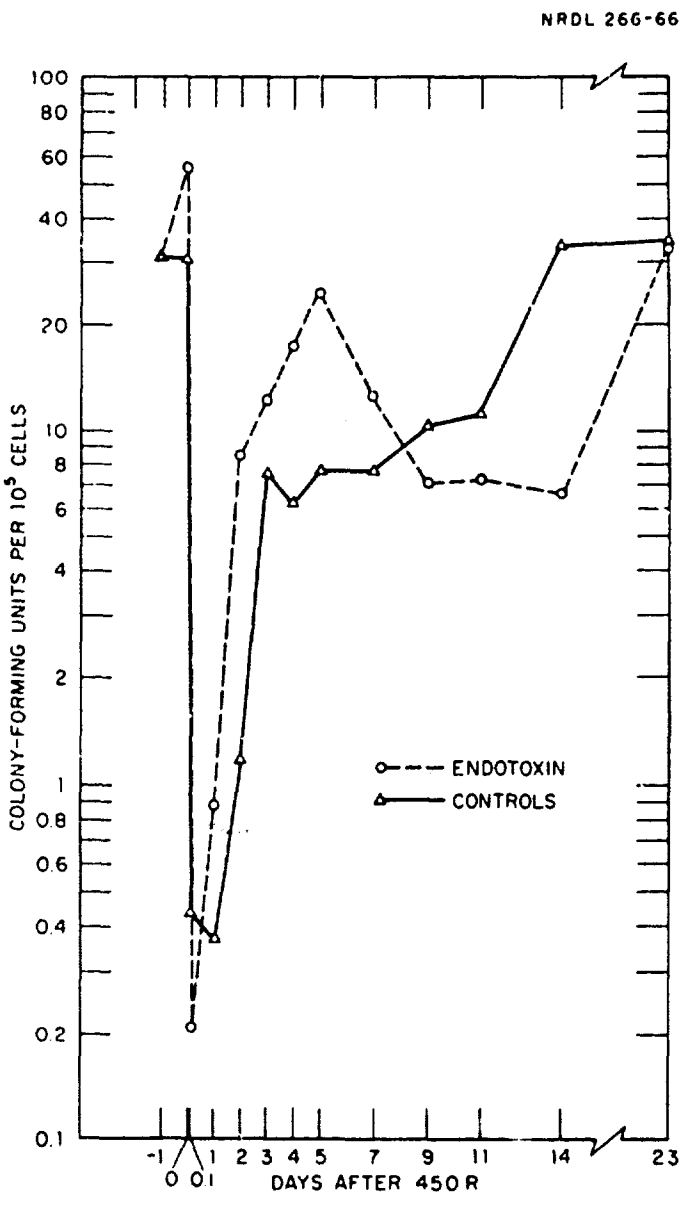

Fig. 2. Changes in femoral bone marrow colony-forming unit content per 10^5 cells after the irradiation of endotoxin-treated and control mice (450 R).

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13 ABSTRACT Radiosensitivity of an animal, in terms of survival or death following midlethal exposure, is thought to be related to the surviving number of hematopoietic stem cells. After a sublethal exposure to radiation an animal's sensitivity to a subsequent exposure (LD_{50}) might also be expected to be related to the number of stem cells which are present at any given time. In the present experiments with mice, we have studied the relationship between split-dose LD_{50} and changes in the numbers of nucleated cells in the femur and the femoral content of colony-forming units (CFU's). These CFU's are proliferative cells in the marrow which when transplanted have the capacity to form nodules in the spleens of supralethally irradiated recipient mice. Many stem cell-like attributes have been conferred to CFU's and the CFU is frequently referred to as a hematopoietic stem cell.

In the present experiments, changes in marrow cellularity and CFU content were studied for three weeks after exposure to 450 R, and preliminary LD_{50}'s were determined at 5 or 14 days. One group of animals was given bacterial endotoxin before the 450 R exposure. During the first week after 450 R, the endotoxin-treated animals showed an accelerated recovery in terms of numbers of nucleated marrow cells and CFU's, and at day 5 the femoral CFU content of the endotoxin-treated animals was ten times as great as that of the controls. However, at this time the LD_{50} of the endotoxin-treated animals was only 50 R higher than the controls. At 14 days, the femoral CFU content of the control animals was approximately four-fold greater than that of the endotoxin-treated group, but the LD_{50}'s of the two groups were quite similar. Therefore, in these experiments with endotoxin-treated and control animals, the femoral CFU content and split-dose radiosensitivity have been found to vary independently.

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